DETECTION OF PREACTIVATED PHAGOCYTES

The present invention relates to an antigen of a phagocyte.

Chronical inflammatory diseases such as, for example, allergic asthma and rheumatoid arthritis, are mediated by inflammation cells such as T-cells and phagocytes. At the location of an inflammation in an organ cytokines are formed. A part of the cytokines diffuses to the peripheral blood where they are involved in the mobilization of new inflammation cells. These inflammation cells are preactivated through an interaction with a cytokine. The extent of preactivation of phagocytes is correlated with the amount of inflammation-promoting cytokines and thus with the extent of the inflammation reaction. Especially for diseases localized in organs such as the lung and the intestines (for example with Crohn's disease) it is physically very difficult or impossible to reliably determine the severity of an inflammation without invasive examination. In addition, invasive examination through a biopt only gives information on the conditions in the biopt itself. Reliable determination of the severity of an inflammation is in particular difficult because until now no antigens specific for a preactivated phagocyte have been found.

The present invention relates to an antigen of a phagocyte, wherein the antigen is recognizable by at least a bacteriophage such as can be isolated from the strains having accession numbers CBS 101481 and 101482.

It has been found that the thus characterized antigen, which is present on the surface of a phagocyte, is specific for a preactivated phagocyte. By establishing the presence of the antigen, and in particular the amount thereof, the presence of an inflammation and its severity can be determined. At least partially purified antigen, or a fragment thereof, may be used for obtaining preactivated phagocyte-recognizing agents.

The invention also relates to a phagocyte-recogniz-

ing agent which recognizes the antigen that is recognized by at least one bacteriophage such as may be isolated from the strains having accession numbers CBS 101481 and 101482.

Such an agent, for example a (monoclonal) antibody, is very useful for establishing the presence of an (organ-bound) inflammation and the severity thereof. In addition, the agent may be used for eliminating preactivated phagocytes from blood, for example, by using a carrier-bound agent which, after contact between carrier and blood, are separated from each other.

An alternative choice is combining the agent with a group deactivating or even killing the (preactivated) phagocyte. Here an antibody provided with a cell-killing unit, for example a RicineB-chain, or a bi-specific antibody provoking the immune-system to eliminate the preactivated phagocyte may be considered. Thus, the group is (chemically) attached to the agent or is part thereof, for example because it has been prepared by genetic engineering. Both chemical coupling as well as genetic engineering are well-known techniques in the art.

Consequently, the invention also relates to a pharmaceutical compound comprising a phagocyte-recognizing deactivating agent together with a pharmaceutically acceptable excipient or carrier.

In view of the first application, establishing the presence of an inflammation, the invention also relates to a method of detecting a preactivated phagocyte, allowing the specific detection of a preactivated phagocyte.

To this end, the method according to the present invention is characterized in that a phagocyte-recognizing agent is contacted with a phagocyte, and a complex formed between the phagocyte-recognizing agent and the phagocyte is detected.

Such a detection of the complex formed may be accomplished according to any of a plurality of methods known in the art. For example, the agent may be fluorescently labelled and binding to the surface of the phagocyte can be established using a fluorescence microscope or flow cytometre (FACS). The label may also be an enzyme, whereby for example

the product of a reaction catalyzed by the enzyme is detected. An assay technique that comes to mind is for example an ELISA. The use of an enzyme is particularly interesting when the agent is a bacteriophage, as the bacteriophage may be genetically engineered to code for this enzyme. Then the agent does not need to be labelled.

According to an interesting embodiment the agent is capable of competing with at least a bacteriophage such as can be isolated from the strains having accession numbers CBS 101481 and 101482, and a complex formed between the phagocyte-recognizing agent and the phagocyte is detected.

Because of the availability of the bacteriophages that can be isolated from the strains mentioned above, it is now possible to screen for further phagocyte-recognizing agents, since the method according to the present invention is also an excellent test for that purpose. Such phagocyte-recognizing agents may, for example, be peptides, including peptidomimetica and peptides with unusual amino acids, or organic compounds which may be prepared using combinatorial chemistry. The use of such a phagocyte-recognizing agent, to which of course also the two bacteriophages belong that may be isolated from the strains mentioned above, makes it possible to establish whether a phagocyte from the blood of a person or other mammal is a preactivated phagocyte.

According to an advantageous embodiment the agent is a fluorescent agent.

This facilitates easy detection of the complex formed, for example, using a fluorescence microscope.

According to an interesting embodiment the agent comprises protein capable of emitting visible fluorescent light, which protein either does not need a prosthetic group, or requires a prosthetic group chosen from a metal ion present in physiological medium. Suitably the fluorescent protein is Green or Blue Fluorescent Protein.

This embodiment is in particular interesting when the agent is a bacteriophage, as the bacteriophage may be genetically engineered to code for this fluorescent protein. Then the agent does not need to be labelled with a fluorescent substance.

Advantageously detection occurs using a Fluorescence-Activated Cell Sorter (FACS).

This method allows for a fast detection and quantification of preactivated phagocytes. This is in particular advantageous when it is important to gain an understanding quickly about the condition of a patient, such as with septic shock, but also for screening a range of compounds, such as those mentioned before, for phagocyte-recognizing properties.

According to an alternative embodiment detection is performed using an ELISA. There use can be made of an antibody (labelled with an enzyme) against the phagocyte-recognizing agent. Again the agent may be (or comprise) a fusion-protein comprising the enzyme.

This method has the advantage that a possibly preactivated phagocyte-containing sample does not need to be fresh. This method also makes it possible to screen a very large number of compounds for their phagocyte-recognizing activity.

The present invention is particularly suited for detecting a preactivated phagocyte, wherein that phagocyte is derived from a person believed to suffer from a condition selected from the group consisting of i) organ-bound inflammatory diseases, such as inflammatory lung diseases (for example allergic asthma, COPD, and cystic fibrosis) and intestinal diseases (for example Colitis ulcerosa, and Crohn's disease); ii) septic shock; iii) allergies; and iv) auto-immune diseases (for example rheumatoid arthritis); as well as from a trauma patient (for example early detection of ARDS); or a person who gas undergone a transplantion (early detection of rejection).

Applicant does not rule out that the method according to the present invention may be used to objectively detect and possibly quantify one or more forms of Repetitive Strain Injury. In the latter case, it might be possible to measure the effect of a treatment with, for example, corticosteroids or of physiotherapy.

Advantageously, blood of a person is lysed with isotonic, cold NH_4Cl-solution, yielding a phagocyte-containing

solution.

Thus it is possible to very quickly prepare a phagocyte-comprising solution suitable for the method according to the present invention, wherein the possibl presence of preactivated phagocytes may be detected.

The present invention will now be elucidated with reference to the following example, wherein

Fig. 1 shows the specific primed neutrophilic granulocytes-recognizing nature of an agent according to the invention;

Fig. 2 shows that this occurs independent of the manner of priming;

Fig. 3 shows the dose-dependency of priming with GM-CSF;

Fig. 4 shows that the epitope recognized by an agent according to the invention is present to an elevated extent on eosinophilic granulocytes derived from a patient having symptomatic allergic asthma;

Fig. 5 shows the absence of an effect of a drug used for the treatment of asthma on the expression of the antigen;

Fig. 6 shows the relationship between the bronchial hyperreactivity in patients suffering from allergic asthma and the expression of the antigen according to the invention on eosinophilic granulocytes; and

Fig. 7 depicts an autoradiogram of an immuno-precipitate containing an antigen according to the invention.

1) PHAGE-LIBRARY WITH ANTIBODY-FRAGMENTS ON THE SURFACE OF THE PHAGES.

By means of the "phage display"-technology, proteins can be expressed on the coat of bacteriophages. In the present case and using DNA genetic-engineering technology, a piece of DNA coding for a part of an antibody-molecule is introduced in the same reading frame as the DNA coding for bacteriophage g3p-coat protein. Thus a g3p-comprising fusion-protein is formed. In a library of such bacteriophages each of the phages possesses a different antibody specificity. To this end, DNA from B-lymphocytes coding for antibody is used in a large number of phages. For the present invention use is

made of the "Phage-antibody library" described by De Kruif et al. (ref. 1) and the American patent application No. 09/085,072, the specification of which is included by reference.

In short, degenerated oligonucleotides were used to add artificial CDR3-areas with a length of 6 to 15 nucleotides to a set of 49 pre-cloned germline VH-genes. Subsequently these in vitro "rearranged" VH-genes were cloned into a collection of pHEN1 vectors derived from phagemid containing seven different light chain V-regions, fused in a reading frame of the gene coding for the phage minor capsid protein-gene III. Introducing these constructs, for example, in E.coli XL-1 bacteria (Stratagene) using a helper-phage (VCSM13, act. No. 200251, Stratagene) results in expression of single chain FV-antibody fragments as gene III fusion-proteins on the surface of the bacteriophage.

A phagemid library was obtained of 1.2 x 10^8 clones.

2) ENRICHING PHAGES IN PREACTIVATED PHAGOCYTE-SPECIFIC BACTERIOPHAGES AND CLONING.

Unprimed leucocytes were isolated applying a generally known technique using isotonic lysis by means of isotonic cold NH_4Cl solution. It is very important to prevent contamination with lipopolysacharid (LPS) by using LPS-free media, as LPS may prime artificially, as a result of which the method may not be performed with success. A part (10^8) of the unprimed leucocyte-population was contacted with the bacteriophage library (10^{11} bacteriophages). After 30 minutes, the leucocytes were spun down (pre-clearing) and not used. The bacteriophages recognizing un-primed leucocytes remain in the supernatant. Subsequently an other part of the leucocytes population is primed with granulocyte macrophage-colony stimulating factor GM-CSF (100 pM, 30 min., 37°C) and subsequently contacted with the previously obtained supernatant containing bacteriophages. Now the desired bacteriophages bind to the leucocytes. The leucocytes were washed and bacteriophages bound to the primed cells were visualized using a two-step staining. First the cells were contacted with a polyclonal antibody recognizing the bacteriophages (anti-M13). Subsequently an antibody labelled

with phyco-erythrin against the anti-M13 polyclonal was used to visualize by means of a FACS the cells that have bound bacteriophages. The FACS was provided with a cell sorter and those leucocytes were isolated which met the following condition: Fluorescent primed eosinophilic granulocytes (see ref. 2). The bacteriophages were eluted from the sorted primed eosinophilic granulocytes (a sub-class of leucocytes) and multiplied as described before. This method (pre-clearing to elution and multiplication) was repeated three times as a result increasingly pure bacteriophage suspensions were obtained. Subsequently 200 bacteriophage clones were multiplied and evaluated using the following procedure and using a FACS.

SELECTION OF BACTERIOPHAGES SPECIFIC FOR PRE-ACTIVATED PHAGOCYTES.

Unprimed leucocytes were isolated as described under 2. A second part of the leucocytes was treated with GM-CSF and fluorescently green labelled with sulfidofluorescein diacetate (SFDA, for labelling procedure see ref. 3). Thereafter the primed fluorescent leucocytes were mixed with unstained unprimed cells in a ratio of 1:1. Subsequently this mixed cell-population was contacted with various bacteriophage clones. The clones of interest had to have the following properties: (i) no expression on lymphocytes (at rest/activated), negative on neutrophilic eosinophilic granulocytes at rest (which can be recognized as non-green granulocytes and with the gates mentioned for eosinophilic granulocytes mentioned in ref. 2), and positive for GM-CSF primed granulocytes (recognizable as green cells in the above gates). Two different bacteriophage clones, as may be isolated from the strains A17 and A27 with accession numbers CBS 101481 and 101482 respectively, showed these characteristics. The strains were deposited on 1 December, 1998 with the CBS (Centraalbureau voor Schimmelcultures, P.O. Box 273, NL-3740 AG, Baarn).

Fig. 1 shows that bacteriophage A17 recognizes neutrophilic granulocytes primed with GM-CSF, whereas non-primed neutrophilic granulocytes are not recognized. In short: Full blood was pre-incubated with buffer or with GM-

CSF (10 pM) for 15 min. at 37°C. Thereafter the red blood-cells were lysed using ice-cold NH_4Cl-solution. Subsequently the white blood-cells were washed and stained using bacteriophage A17 and analyzed using a flow-cytometer. The neutrophilic granulocytes were identified by their unique forward and sidewards light-scattering characteristics. The experiment shown is representative of 25 different experiments.

Fig. 2 shows the relative fluorescence-intensity of unprimed neutrophilic granulocytes and neutrophilic granulocytes primed with GM-CSF (100 pM, 30 min. 37°C) or TNFα (100 IU/ml; 20 min.; 37°C). It is clearly shown that both ways of priming result in an increased presence of the epitope recognized by bacteriophage A27. In short: full blood was treated with GM-CSF (100 pM), TNFα (100 IU/ml, IL-5 (100 pM) or buffer for 15 min. at 37°C. Subsequently, the red blood cells were lysed (using ice-cold NH_4Cl-solution), and washed and stained with A27(A) and A17(B) as described above. The values are given as averages ± SE (n=24). Values having a * are significantly different from the buffer-control ($p<0,001$). Comparable results were obtained for human monocytes (results not shown).

Fig. 3 shows the dose-dependency of priming with GM-CSF for the bacteriophage-strains A17 and A27. In short: Full blood was treated with various concentrations GM-CSF or buffer during 15 min. at 37°C. Subsequently the red cells were lysed (using ice-cold NH_4Cl-solution), and washing and staining occurred with A27(A) and A17(B) as described above. The values are given as averages ± SE (n=10).

Fig. 4 shows that the epitope recognized by bacteriophage A17 is present to an increased extent on eosinophilic granulocytes obtained from a patient having symptomatic allergic asthma. This experiment shows that eosinophilic granulocytes in the blood of patients having symptomatic asthma have a preactivated phenotype. The data of the patient-cells were compared with cells from the blood (obtained at the same day) of a normal donor before and after treatment in vitro with GM-CSF (100 pM). The experiment shown is representative of at least 15 further experiments. Similar

results were obtained for COPD (Chronic Obstructive Pulmonary Disease, a smoking-related respiratory disease). With COPD it is the neutrophilic granulocytes showing the elevated expression of the antigen, whereas with asthma the eosinophilic granulocytes play the leading part (results not shown).

Fig. 5 shows the suitability of the antigen according to the present invention: glucocorticosteroids, such as dexamethasone or prednisone, used for the treatment, have no influence on the (in vitro) expression of the antigen. That is, the reduction of the expression in vivo is the result of the repression of the inflammation. In short: Stem-cells were obtained from blood from the umbilical cord and cultured under conditions leading to the terminal differentiation to neutrophilic granulocytes. These were primed by cytokines present in the medium, necessary for differentiation. Addition of dexamethasone (1 μM) to the culture had no influence on the expression of the antigen.

Fig. 6 shows the relation between bronchial hyperreactivity and the expression of the antigen on eosinophilic granulocytes such as measured with A27. The measurement of bronchial hyperreactivity is done by having a patient inhale histamine (or a compound having a similar effect) and to measure at which concentration (mg/ml) the lung-capacity is reduced by 20%. This measurement is very taxing for patients and should in fact not be used with certain patient-groups, such as the elderly and very small children. From Fig. 6 it appears that there is an excellent correlation between patients having a low histamine threshold and the expression of the antigen. This means that the very taxing, time-consuming test may be replaced by a simple quick measurement on a blood-sample.

Until now no marker has been found for COPD-patients correlating with the tightness of the chest experienced by the patient (Borg score). The antigen according to the invention recognized by bacteriophage A27, however, results in a very workable correlation with the Borg score ($r=0.65$, $p<0.001$. Results not shown).

Fig. 7 shows an autoradiograph of an SDS-PAGE gel in

which a specifically-recognized antigen is visible. Isolated human neutrophilic granulocytes primed with FMLP (the tripeptide formyl-methionyl-leucyl-phenylalanide) were surface-labelled with ^{125}I (Iodogen-method). Subsequently bacteriophage A17 and A27 and constructs thereof (ScFv-fragments made in accordance with ref. 1 and humanized antibody with the antigen-recognizing sequences of bacteriophage A17 (ref. 4)) were contacted on ice for 90 min. with the radioactively labelled granulocytes. After washing (2x) the bound bacteriophages and constructs thereof were (reversibly) cross-linked, in accordance with the instructions of the manufacturer with BTSSP (Pierce, Rockford, IL) to granulocytes. After lysis of the granulocytes an immuno-precipitation was performed (2-4 hours at 4°C). This was done for the bacteriophages with anti-M13 antibodies (Pharmacia) bound to Protein A-agarose. For the ScFv-fragments this was done with anti-myc antibodies, and for humanized IgG directly with Protein A-agarose. Washing occurred three times with lysis buffer. The cross-linked antibodies and antigens were separated by reduction and immediately applied to a 10% SDS-PAA gel. Specifically immuno-precipitated proteins are indicated with an arrow (control not shown). The importance of the use of constructs apart from the bacteriophage is that especially non-binding parts of the antibodies show a-specific adsorption. Using different antibodies, with various non-binding parts increases the possibility of differentiating between specific and possibly present a-specific bands.

References

Ref. 1 The "Phage antibody library" used is described by De Kruif et al. (Kruif et al., 1995. Selection and application of human scFv antibody fragments from a semi-synthetic phage antibody display library with "designed" CDR3 regions, J.Mol. Biol., 248, 97 - 105).

Ref. 2 Mengelers, H.J.J., Maikoe, T., Brinkman, L., Hooibrink, B., Lammers, J.W.J. and Koenderman, L. (1994). Immunophenotype of eosinophils recovered from blood and bronchoalveolar lavage of allergic asthmatics. Am. J. Respir. Crit. Care med. 149: 345 - 351).

Ref. 3 Mengelers, H.J.J., Maikoe, T., Brinkman, L., Hooibrink, B., Lammers, J.W.J. and Koenderman, L. (1995). Cognate interaction between human lymphocytes and eosinophils is mediated by beta2-integrins and very late antigen-4. J. Lab. Clin. Med. 126: 261 - 268).

Ref. 4 Huls et al. Nature Biotechnology, 17: page 276 - 281 (1999)).

The description of these references is incorporated by reference.